IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF PROTEINS, EXPRESSED IN THE *IXODES RICINUS* SALIVARY GLANDS.

Cross reference to related applications

This application is a Continuation-in-Part of PCT Application Number PCT/BE00/00061 filed on June 6, 2000, the disclosure of which is incorporated herein by reference in its entirety. PCT/BE00/00061 claims priority to GB9913425.6, filed June 9, 1999, the disclosure of which is incorporated herein by reference in its entirety.

Field of the invention

The present invention is related to the molecular characterization of DNA sequences, which encode proteins expressed in the salivary glands of the *Ixodes ricinus* arthropod tick. These proteins are involved in the complex mechanism of interaction between this arthropod and its mammalian host. The invention relates to newly identified polynucleotides, polypeptides encoded by them and the use of such polynucleotides and polypeptides, and to their production.

Background of the invention

Ticks are hematophagous arthropods that feed on a wide diversity of hosts {Sauer, Annu. Rev. Entomol, 1995}. Unlike this group of arthropods, the Ixodid adult female ticks have the characteristics to ingest blood for an extended period of over 2 weeks.

Completion of the blood meal is dependent on the relationships of ticks with hosts species {Brossard Med. Vet. Entomol 1997}. Resistance to tick infestation implicates both innate and acquired immunity, and is characterized by reduced feeding, molting and mating capabilities that may lead to the death of the parasite. Acquired immunity of resistant hosts is mediated by a polarized Th1-type immune response, involving IFN-γ production and delayed type hypersensitivity reaction {Allen JR, Int. J. Parasitol. 1973}{Ganapamo, Immunol. 1995}.

Some hosts are unable to counteract the tick infestation {Ganapamo et al, 1995}. Indeed, during their blood meal, ticks circumvent host defences via pharmacologically active components secreted in their saliva. These factors can modulate both the innate and the acquired immunity of the host. In this way, the leukocyte responsiveness is modified during tick feeding {Ribeiro, Exp. Parasitol. 1987}{Kubes, Immunol. 1994}. For example, cytokines production is modulated, inducing a polarised Th2 immune response {Ganapamo, Immunol. 1996} {Kopecky, Parasite Immunol. 1998}.

Therefore, the complex tick-host molecular interaction can be considered as a balance between host defences raised against the parasite and the tick evasion strategies, facilitating feeding for an extended period.
Although, there is extensive information about the effects of tick bioactive factors on host immune defences, little is known about the mechanisms of their actions. However, it has been observed that a wide range of new proteins is expressed during the blood meal {Wang, Parasitol. 1994}. Several of

them might be essential for the completion of the tick feeding process.

Summary of the invention

The present invention is related to a new isolated and purified polynucleotide obtained from tick salivary gland and presenting more than 75% identity with at least one nucleotide sequence selected from the group consisting of SEQ.ID.NO.1, SEQ.ID.NO.2, SEQ.ID.NO.3, SEQ.ID.NO.4, SEQ.ID.NO.5, SEQ.ID.NO.6, SEQ.ID.NO.7, SEQ.ID.NO.9, SEQ.ID.NO.10, SEQ.ID.NO.11, SEQ.ID.NO.12, SEQ.ID.NO.13, SEQ.ID.NO.14, SEQ.ID.NO.15, SEQ.ID.NO.16, SEQ.ID.NO.17, SEQ.ID.NO.19, SEQ.ID.NO.20, SEQ.ID.NO.21, SEQ.ID.NO.22, SEQ.ID.NO.23, SEQ.ID.NO.24, SEQ.ID.NO.25, SEQ.ID.NO.26, SEQ.ID.NO.28, SEQ.ID.NO.29, SEQ.ID.NO.30, SEQ.ID.NO.31, SEQ.ID.NO.33 or a sequence complementary thereto, or a fragment thereof, as defined hereafter.

Preferably, the polynucleotide of claim 1, which presents at least 80% identity with at least one of said nucleotide sequences, more preferably at least 90% identity, more preferably with at least 95% identity, and even at least about 98 to 99% identity.

Preferably, the polynucleotide of claim 1, which presents at least 99% identity with at least one of said nucleotide sequences.

The present invention is also related to a polypeptide encoded by the polynucleotide of the present invention or a biologically active fragment or portion thereof.

Said polypeptide may be modified by or linked to at least one substitution group, preferably selected from

the group consisting of amide, acetyl, phosphoryl, and/or glycosyl groups.

Moreover, said polypeptide may take the form of a "mature" protein.

It may also be part of a larger protein or part of a fusion protein.

Preferably, the polypeptide of the present invention further includes at least one additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which help in purification such as multiple histidine residues, or additional sequences for stability during production of recombinant molecules.

Another object of the present invention concerns a variant of the polynucleotide or the polypeptide of the present invention, a precise definition of this term being given hereafter.

Preferably, said variant varies from the referent by conservative amino acid substitutions.

Preferably, at least one residue is substituted in said variant with another residue of similar characteristics.

Advantageously, the substitutions in said variant are among Ala, Val, Leu and Ile; among Ser and Thr, among the acidic residues Asp and Glu; among Asn and Gln; among the basic residues Lys and Arg; or among aromatic residues Phe and Tyr.

Preferably, in the variant of the present invention, several amino acids are substituted, deleted or added in any combination.

Preferably, 5-10, more preferably 1-5, more preferably 1-2 amino acids are substituted, deleted or added in any combination, in said variant.

Said variant may be a naturally occurring allelic variant of an *Ixodes ricinus* salivary gland polypeptide present in *Ixodes ricinus* salivary glands.

The present invention is also related to a recombinant vector comprising at least one element selected from the polynucleotide, the polypeptide, and the variant of the present invention or fragments thereof.

Another object of the present invention concerns a cell transfected by or comprising the recombinant vector according to the invention.

The present invention further includes an inhibitor directed against said polynucleotide, polypeptide, or variant.

Said inhibitor is preferably an antibody or an hypervariable portion thereof.

The present invention is also related to an hybridoma cell line expressing said inhibitor.

Another object of the present invention concerns a pharmaceutical composition comprising an adequate pharmaceutical carrier and an element selected from the group consisting of said polynucleotide, polypeptide, variant, vector, cell, inhibitor or a mixture thereof.

Preferably, said pharmaceutical composition presents anti-coagulant properties and advantageously contains at least one polynucleotide selected from the group consisting of SEQ.ID.NO.7, SEQ.ID.NO.17, and SEQ.ID.NO.26, and fragments thereof or contains at least one polypeptide encoded by said polynucleotides or fragments thereof.

Preferably, the pharmaceutical composition presents immunomodulatory properties, and contains at least one polynucleotide selected from the group consisting of SEQ.ID.NO.12, SEQ.ID.NO.21, SEQ.ID.NO.26, and SEQ.ID.NO.31, and fragments thereof, or contains at least one polypeptide encoded by said polynucleotides or fragments thereof.

Another object of the invention is an immunological composition or vaccine for inducing an immunological response in a mammalian host to a tick salivary gland polypeptide which comprises at least one element of the group consisting of

a) a polynucleotide of tick salivary glands according to the invention;

b) a polypeptide of tick salivary glands according to the invention;

c) a variant according to the invention;

d) epitope-bearing fragments, analogs, outer-membrane vesicles or cells (attenuated or otherwise) of components a) or b) or c);

e) possibly a carrier.

The present invention is also related to a method for treating or preventing a disease affecting a mammal, said method comprising the step of administrating to said mammal a sufficient amount of the pharmaceutical composition or the immunological composition or vaccine according to the invention, in order to prevent or cure either the transmission of pathogenic agents by tick, especially by *Ixodes ricinus*, or the symptoms of diseases induced by tick or pathogenic agents transmitted by tick.

The present invention is also related to the use of the pharmaceutical composition or the immunological

composition or vaccine according to the invention for the manufacture of a medicament in the treatment and/or prevention of diseases induced by tick or pathogenic agents transmitted by tick, especially by *Ixodes ricinus*.

Advantageously, said medicament may be used in transplantation, in rheumatology, but also in general treatment.

Finally, another object of the invention is a diagnostic kit for detecting a disease or susceptibility to a disease induced or transmitted by tick, especially *Ixodes ricinus*, which comprises:

a) at least one tick salivary gland polynucleotide of the invention, or a fragment thereof;

b) or at least one nucleotide sequence complementary to that of a);

c) or at least one tick salivary gland polypeptide, of the invention or a fragment thereof;

d) or at least one variant according to the invention or a fragment thereof

e) or an inhibitor of the invention;

f) or a phage displaying an antibody of the invention whereby a), b), c), d), e), f) may comprise a substantial component.

Brief description of the drawings

Figure 1 presents results of RACE assay (Frohman et al., 1995) specific to SEQ.ID.NO.17 and SEQ.ID.NO.26. The reverse transcription step was carried out using 10 ng of mRNAs extracted from salivary gland of engorged ticks. The brightest bands represent the cDNA fragments corresponding to the 3′ end of the targeted mRNA.

The amplified products were subjected to agarose gel electrophoresis followed by staining the DNA fragments by ethidium bromide. Arrows indicate the position of the expected amplified products.

Figure 2 represents differential expression analysis of the 5 full-length selected cDNAs and 9 cDNA fragments isolated in the subtractive library. PCR assays were carried out using as DNA template cDNAs obtained from a reverse transcription procedure on mRNAs extracted from salivary glands either of engorged (E) or of unfed (UF) ticks. These RNA messengers were also used as template in reverse transcription assays. Ten microliter of both PCR and RT-PCR mixture were subjected to agarose gel electrophoresis and ethidium bromide staining for the detection of amplified DNA products. [++] strongly positive; [+] positive; [-] negative.

Figure 3 relates to the detection of the native Iris (formerly named SEQ.ID.NO.26) protein by western blots using anti-rIris/MBP serum were realised on 5 day fed tick saliva.

Figure 4 represents results obtained by confocal microscopy of female *I. ricinus* salivary glands. A. Negative control corresponding to 5 day fed tick salivary glands incubated only with the secondary antibody. Unfed (B), 3 day (C) and 5 day (D) fed tick salivary glands incubated with the anti-rIris/MBP serum.

Figure 5 represents in vitro proliferation assays of Balb/c spleen cells stimulated with ConA. Spleen cells were incubated with only ConA (N.S.) or with various dilutions of rIris/His and NEG cellular extracts. Tritiated

thymidine incorporation was determined by liquid scintillation counting (10^{-3} c.p.m. +/- S.D.).

Figure 6 presents Balb/c tick-specific lymph nodes cells proliferation assays. Lymph nodes cells were isolated from a mouse pre-infested with *I. ricinus* nymphs. Cells were stimulated with various dilutions of rIris/His and NEG cellular extracts. Tritiated thymidine incorporation was determined by liquid scintillation counting (10^{-3} c.p.m.).

Figure 7 refers to IFN-γ and IL-10 ELIspot of human PBMCs. The number of activated cells producing the cytokines upon treatment with PHA or LPS was evaluated (spots/10^6 cells +/- S.D.). Activated cells were counted after treatment with rIris/His or NEG cellular extracts. A positive control was realised by stimulating the cells only with the activator (PHA or LPS).

Figure 8 relates to the IFN-γ and IL-5 production by human PBMCs. Cells were incubated with only the activator (N.S.) or with various dilutions of rIris/His and NEG cellular extracts. The production of IFN-γ and IL-5 (pg/ml +/- S.D.) was evaluated upon treatment with CD3/CD28 or PPD.

Figure 9 represents IFN-γ production by human PBMCs stimulated with CD3/CD28. The cells were stimulated with NEG and rIris/His cellular extracts at a 1:12.5 dilution. All the assays were realized by stimulating the cells: only with CD3/CD28 (P.C.), with CD3/CD28 in the presence of NEG cellular extract (NEG), or with CD3/CD28 in the presence of rIris/His cellular extract (rIris/His). A. CD3/CD28 stimulated cells were incubated with rIris/His cellular extract in the presence of various dilutions of either anti-rIris/MBP serum (Anti-rIris/MBP+rIris/His) or a

non-specific serum (ns Ab+rIris/His). B. CD3/CD28-stimulated cells were incubated with 400 nM CsA (CsA), with 400 nM CsA in the presence of anti-rIris/MBP serum (CsA + Anti-rIris/MBP), or with 400 nM CsA and the non-specific serum (CsA + NS AB); both antisera were used at a 1:250 dilution. C. CD3/CD28 stimulated PBMCs were also incubated with purified NEG (pNEG) or rIris/His (pIris/His) protein, and with purified NEG or rIris/His proteins in the presence of anti-rIris/MBP serum at a 1:250 dilution (pNEG + Anti-rIris/MBP and pIris/His + Anti-rIris/MBP).

Definitions

"Putative anticoagulant, anti-complementary and immunomodulatory" polypeptides refer to polypeptides having the amino acid sequence encoded by the genes indicated in the table. These present homologies with anticoagulant, anti-complementary and immunomodulatory polypeptides already existing in databases. These polypeptides belong to the Class I and Class II sequences (see table).

"Putative anticoagulant, anti-complementary and immunomodulatory" cDNAs refer to polynucleotides having the nucleotide sequence described in the table, or allele variants thereof and/or their complements. These present homologies with anticoagulant, anti-complementary and immunomodulatory polynucleotides already existing in databases. These cDNAs belong to the Class I and Class II sequences (see table)

Some polypeptide or polynucleotide sequences present low or no homologies with already existing polypeptides or polynucleotides in databases. These belong to the Class III (see table).

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslational natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a hem moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-linkings,

formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Comany, New York, 1993 and Wolt, F., Posttranslational Protein Modifications : Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", *Meth Enzymol* (1990) 182 : 626-646 and Rattan *et al*, "Protein Synthesis : Posttranslational Modifications and Aging", *Ann NY Acad Sci* (1992) 663 : 48-62.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double- stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "Polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "Polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability

or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "Polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions (preferably conservative), additions and deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic

variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis. Variants should retain one or more of the biological activities of the reference polypeptide. For instance, they should have similar antigenic or immunogenic activities as the reference polypeptide. Antigenicity can be tested using standard immunoblot experiments, preferably using polyclonal sera against the reference polypeptide. The immunogenicity can be tested by measuring antibody responses (using polyclonal sera generated against the variant polypeptide) against purified reference polypeptide in a standard ELISA test. Preferably, a variant would retain all of the above biological activities.

"Identity" is a measure of the identity of nucleotide sequences or amino acid sequences. In general, the sequences are aligned so that the highest order match is obtained. "Identify" *per se* has an art-recognized meaning and can be calculated using published techniques. See, e.g.: (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, 1988; BIOCOMPUTING : INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, 1993; COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds, Humana Press, New Jersey, 1994; SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heijne, G., Academic Press, 1987; and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds, M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled

artisans (Carillo, H., and Lipton, D., *SIAM J Applied Math* (1998) 48 : 1073). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited **to** those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H., and Lipton, D., *SIAM J Applied Math* (1988) 48 : 1073. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., *et al.*, *J Molec Biol* (1990) 215 : 403). Most preferably, the program used to determine identity levels was the GAP program, as was used in the Examples hereafter.

As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include an average up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5′ or 3′ terminal positions of the reference nucleotide sequence or anywhere between those terminal

positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

Fragments of *I. ricinus* salivary gland polypeptides are also included in the present invention. A fragment is a polypeptide having an amino acid sequence that is the same as a part, but not all, of the amino acid sequence of the aforementioned *I. ricinus* salivary gland polypeptides. As with *I. ricinus* salivary gland polypeptides, fragment may be "free-standing" or comprised within a larger polypeptide of which they form a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments from about amino acid number 1-20, 21-40, 41-60, 61-80, 81-100, and 101 to the end of the polypeptide. In this context "about" includes the particularly recited ranges larger or smaller by several, 5, 4, 3, 2 or 1 amino acid at either extreme or at both extremes.

Preferred fragments include, for example, truncated polypeptides having the amino acid sequence of the *I. ricinus* salivary gland polypeptides, except for deletion of a continuous series of residues that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus and / or transmembrane region or deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Also preferred are fragments characterised by structural or functional attributes such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet forming regions, turn and turn-forming regions, coil

and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Other preferred fragments are biologically active fragments. Biologically active fragments are those that mediate *I. ricinus* salivary gland protein activity, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Also included are those that are antigenic or immunogenic in an animal or in a human.

Examples

Example 1: Characterization of the induced genes.

Genes are induced in the salivary glands of *Ixodes ricinus* during the slow-feeding phase of the blood meal. The cloning of these genes was carried out by setting up two complementary DNA (cDNA) libraries. The first one is a subtractive library based on the methodology described by Lisitsyn *et al.* (*Science* 259, 946-951,1993) and improved by Diatchenko *et al.* (*Proc. Natl. Acad. Sci. USA* 93, 6025-6030, 1996). This library cloned selectively induced mRNA during the tick feeding phase. The second library is a full-length cDNA library, which was constructed by using the basic property of mRNAs (presence of a polyA tail in its 3'end and a cap structure in its 5' end). This cDNA library permitted the cloning of full-length cDNAs, corresponding to some incomplete cDNA sequences identified in the subtractive cDNA library.

The subtractive library was set up by subtracting uninduced-cDNAs (synthesized from mRNAs equally

expressed in the salivary glands of both unfed and engorged ticks) from induced-cDNAs (synthesised from mRNAs differentially expressed in the salivary gland at the end of the slow-feeding phase). The induced-cDNAs was digested by a restriction enzyme, divided into two aliquots, and distinctively modified by the addition of specific adapters. As for the induced-cDNAs, the uninduced cDNAs was also digested by the same restriction enzyme and then mixed in excess to each aliquot of modified induced-cDNA. Each mixture of uninduced-/induced-cDNAs was subjected to a denaturation step, immediately followed by an hybridisation step, leading to a capture of homologous induced-cDNAs by the uninduced-cDNA. Each mixture was then mixed together and subjected again to a new denaturation/hybridisation cycle. Among the hybridised cDNA molecules, the final mixture comprises induced-cDNAs with different adapters at their 5' and 3' end. These relevant cDNAs were amplified by polymerase chain reaction (PCR), using primers specific to each adapter located at each end of the cDNA molecules. The PCR products were then ligated into the pCRII™ vector by A-T cloning and cloned in an TOP-10 *E. coli* strain. The heterogeneity of this subtractive library was evaluated by sequencing 96 randomly chosen recombinant clones. The "induced" property of these cDNA sequences was checked by reverse transcription-PCR (RT-PCR) on mRNA extracted from salivary glands of engorged and unfed ticks. Finally, the full-length induced-cDNA was obtained by screening the full-length cDNA library using, as a probe, some incomplete induced-cDNAs isolated from the subtractive library. These full-length induced DNA molecules were sequenced and compared to known polypeptide

and polynucleotide sequences existing in the EMBL/GenBank databases.

The full-length cDNA library was set up by using the strategy developed in the "CapFinder PCR cDNA Library Construction Kit" (Clontech). This library construction kit utilises the unique CapSwitch™ oligonucleotide (patent pending) in the first-strand synthesis, followed by a long-distance PCR amplification to generate high yields of full-length, double-stranded cDNAs. All commonly used cDNA synthesis methods rely on the ability of reverse transcriptase to transcribe mRNA into single stranded DNA in the first-strand reaction. However, because the reverse transcriptase cannot always transcribe the entire mRNA sequence, the 5' ends of genes tend to be under-represented in cDNA population. This is particularly true for long mRNAs, especially if the first-strand synthesis is primed with oligo(dT) primers only, or if the mRNA has a persistent secondary structure. Furthermore, the use of T4 DNA polymerase to generate blunt cDNA ends after second-strand synthesis commonly results in heterogeneous 5' ends that are 5-30 nucleotides shorter than the original mRNA (D'Alessio, 1988). In the CapFinder cDNA synthesis method, a modified oligo(dT) primer is used to prime the first-strand reaction, and the CapSwitch oligonucleotide acts as a short, extended template at the 5' end for the reverse transcriptase. When the reverse transcriptase reaches the 5' end of the mRNA, the enzyme switches templates and continues replicating to the end of the CapSwitch oligonucleotide. This switching in most cases occurs at the 7-methylguanosine cap structure, which is present at the 5' end of all eukaryotic mRNAs (Furuichi & Miura, 1975). The resulting

full-length single stranded cDNA contains the complete 5' end of the mRNA as well as the sequence complementary to the CapSwitch oligonucleotide, which then serves as a universal PCR priming site (CapSwitch anchor) in the subsequent amplification. The CapSwitch-anchored single stranded cDNA is used directly (without an intervening purification step) for PCR. Only those oligo(dT)-primed single stranded cDNAs having a CapSwitch anchor sequence at the 5' end can serve as templates and be exponentially amplified using the 3' and 5' PCR primers. In most cases, incomplete cDNAs and cDNA transcribed from poly-A RNA will not be recognised by the CapSwitch anchor and therefore will not be amplified.

At the end of these reactions, the full-length cDNA PCR products was ligated into the pCRII cloning vector (Invitrogen) and used for the transformation of XL2 *E. coli* strain. The full-length cDNA library was then screened by using, as a probe, the incomplete induced-cDNAs isolated from the subtractive library.

Ninety-six clones of subtractive library were randomly sequenced, and their DNA and amino acid translated sequences were compared to DNA and protein present in databases. Among these, 27 distinct family sequences were identified, and 3 of them were selected for further characterization of their corresponding full-length mRNA sequence. These 3 sequences matched the sequence of i) the human tissue factor pathway inhibitor (TFPI), ii) the human thrombin inhibitor gene, and iii) a snake venom zinc-dependent metalloprotease protein. These genes encode proteins that could be involved in the inhibition of the blood coagulation. The other 24 family sequences presented low or no homologies with polynucleotide and polypeptide

sequences existing in databases. Screening of the full-length cDNA library using oligonucleotide probes specific to the 3 previously selected subtractive clones lead to the recovery of the corresponding full-length cDNAs. Random screening of this library led to the selection of 2 other clones. One is closely homologous to an interferon-like protein, whereas the other shows homologies to the *Streptococcus equi* M protein, an anti-complement protein.

These polypeptides expressed by *I. ricinus* salivary glands include the polypeptides encoded by the cDNAs defined in the tables, and polypeptides comprising the amino acid sequences which have at least 75 % identity to that encoded by the cDNAs defined in the tables over their complete length, and preferable at least 80 % identity, and more preferably at least 90 % identity. Those with about 95-99 % are highly preferred.

The *I. ricinus* salivary gland polypeptides may be in the form of the "mature" protein or may be a part of a larger protein such as a fusion protein. It may be advantageous to include an additional amino acid sequence, which contains secretory or leader sequences, pro-sequences, sequences which help in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

Preferably, all of these polypeptide fragments retain parts of the biological activity (for instance antigenic or immunogenic) of the *I. ricinus* salivary gland polypeptides, including antigenic activity. Variants of the defined sequence and fragments also form part of the present invention. Preferred variants are those that vary from the referents by conservative amino acid substitutions - i.e.,

those that substitute a residue with another of like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination. Most preferred variants are naturally occurring allelic variants of the *I. ricinus* salivary gland polypeptide present in *I. ricinus* salivary glands.

The *I. ricinus* salivary gland polypeptides of the invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinant polypeptides, synthetic polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

The *I. ricinus* salivary gland cDNAs (polynucleotides) include isolated polynucleotides which encode *I. ricinus* salivary gland polypeptides and fragments thereof, and polynucleotides closely related thereto. More specifically, *I. ricinus* salivary gland cDNAs of the invention include a polynucleotide comprising the nucleotide sequence of cDNAs defined in the table, encoding a *I. ricinus* salivary gland polypeptide. The *I. ricinus* salivary gland cDNAs further include a polynucleotide sequence that has at least 75% identity over its entire length to a nucleotide sequence encoding the *I. ricinus* salivary gland polypeptide encoded by the cDNAs defined in the tables, and a polynucleotide comprising a nucleotide sequence that is at least 75% identical to that of the cDNAs defined in the

tables, in this regard, polynucleotides at least 80% identical are particularly preferred, and those with at least 90% are especially preferred. Furthermore, those with at least 95% are highly preferred and those with at least 98-99% are most highly preferred, with at least 99% being the most preferred. Also included under *I. ricinus* salivary gland cDNAs is a nucleotide sequence, which has sufficient identity to a nucleotide sequence of a cDNA defined in the tables to hybridise under conditions usable for amplification or for use as a probe or marker. The invention also provides polynucleotides which are complementary to such *I. ricinus* salivary gland cDNAs.

These nucleotide sequences defined in the tables as a result of the redundancy (degeneracy) of the genetic code may also encode the polypeptides encoded by the genes defined in the tables.

When the polynucleotides of the invention are used for the production of an *I. ricinus* salivary gland recombinant polypeptide, the polynucleotide may include the coding sequence for the mature polypeptide or a fragment thereof, by itself; the coding sequence for the mature polypeptide or fragment in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro-or preproprotein sequence, or other fusion peptide portions. For example, a marker sequence, which facilitates purification of the fused polypeptide can be encoded. Preferably, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al*, *Proc Natl Acad Sci USA* (1989) 86:821-824, or is an HA tag, or is glutathione-s-transferase. The polynucleotide may also contain non-coding

5′ and 3′ sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Further preferred embodiments are polynucleotides encoding *I. ricinus* salivary gland protein variants comprising the amino acid sequence of the *I. ricinus* salivary gland polypeptide encoded by the cDNAs defined by the table respectively in which several, 10-25, 5-10, 1-5, 1-3, 1-2 or 1 amino acid residues are substituted, deleted or added, in any combination. Most preferred variant polynucleotides are those naturally occurring *I. ricinus* sequences that encode allelic variants of the *I. ricinus* salivary gland proteins in *I. ricinus*.

The present invention further relates to polynucleotides that hybridise preferably stringent conditions to the herein above-described sequences. As herein used, the term "stringent conditions" means hybridisation will occur only if there is at least 80%, and preferably at least 90%, and more preferably at least 95%, yet even more preferably 97-99% identity between the sequences.

Polynucleotides of the invention, which are identical or sufficiently identical to a nucleotide sequence of any gene defined in the table or a fragment thereof, may be used as hybridisation probes for cDNA clones encoding *I. ricinus* salivary gland polypeptides respectively and to isolate cDNA clones of other genes (including cDNAs encoding homologs and orthologs from species other than *I. ricinus*) that have a high sequence similarity to the *I. ricinus* salivary gland cDNAs. Such hybridisation techniques are known to those of skill in the art. Typically these nucleotide sequences are 80% identical, preferably 90%

identical, more preferably 95% identical to that of the referent. The probes generally comprise at least 15 nucleotides, preferably, at least 30 nucleotides or at least 50 nucleotides. Particularly preferred probes range between 30 and 50 nucleotides. In one embodiment, to obtain a polynucleotide encoding *I. ricinus* salivary gland polypeptide, including homologues and orthologues from species other than *I. ricinus*, comprises the steps of screening an appropriate library under stringent hybridisation conditions with a labelled probe having a nucleotide sequence contained in one of the gene sequences defined by the table, or a fragment thereof; and isolating full-length cDNA clones containing said polynucleotide sequence. Thus in another aspect, *I. ricinus* salivary gland polynucleotides of the present invention further include a nucleotide sequence comprising a nucleotide sequence that hybridise under stringent condition to a nucleotide sequence having a nucleotide sequence contained in the cDNAs defined in the tables or a fragment thereof. Also included with *I. ricinus* salivary gland polypeptides are polypeptides comprising amino acid sequences encoded by nucleotide sequences obtained by the above hybridisation conditions (conditions under overnight incubation at 42°C in a solution comprising: 50% formamide, 5xSSC (150mM NaCl, 15mM trisodium citrate), 50mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1xSSC at about 65°C).

The polynucleotides and polypeptides of the present invention may be employed as research reagents and

materials for the development of treatments and diagnostics tools specific to animal and human disease.

This invention also relates to the use of *I. ricinus* salivary gland polypeptides, or *I. ricinus* salivary gland polynucleotides, for use as diagnostic reagents.

Materials for diagnosis may be obtained from a subject's cells, such as from blood, urine, saliva, tissue biopsy.

Thus in another aspect, the present invention relates to a diagnostic kit for a disease or susceptibility to a disease which comprises:

(a) an *I. ricinus* salivary gland polynucleotide, preferably the nucleotide sequence of one of the gene sequences defined by the table, or a fragment thereof;

(b) a nucleotide sequence complementary to that of(a);

(c) an *I. ricinus* salivary gland polypeptide, preferably the polypeptide encoded by one of the gene sequences defined in the table, or a fragment thereof;

(d) an antibody to an *I. ricinus* salivary gland polypeptide, preferably to the polypeptide encoded by one of the gene sequences defined in the table; or

(e) a phage displaying an antibody to an *I. ricinus* salivary gland polypeptide, preferably to the polypeptide encoded by one of the cDNAs sequences defined in the table.

It will be appreciated that in any such kit, (a), (b), (c), (d) or (e) may comprise a substantial component.

Another aspect of the invention relates to a method for inducing an immunological response in a mammal which comprises inoculating the mammal with *I. ricinus* salivary gland polypeptide or epitope-bearing fragments,

analogues, outer-membrane vesicles or cells (attenuated or otherwise), adequate to produce antibody and/or T cell immune response to protect said animal from bacteria and viruses which could be transmitted during the blood meal of *I. ricinus* and related species. In particular the invention relates to the use of *I. ricinus* salivary gland polypeptides encoded by the cDNAs defined in the tables. Yet another aspect of the invention relates to a method of inducing immunological response in a mammal which comprises, delivering *I. ricinus* salivary gland polypeptide via a recombinant vector directing expression of *I. ricinus* salivary gland polynucleotide *in vivo* in order to induce such an immunological response to produce antibody to protect said animal from diseases transmitted by *I. ricinus* ticks or other related species (Lyme disease, tick encephalitis virus disease, ….).

A further aspect of the invention relates to an immunological composition or vaccine formulation which, when introduced into a mammalian host, induces an immunological response in that mammal to a *I. ricinus* salivary gland polypeptide wherein the composition comprises a *I. ricinus* salivary gland cDNA, or *I. ricinus* salivary gland polypeptide or epitope-bearing fragments, analogs, outer-membrane vesicles or cells (attenuated or otherwise). The vaccine formulation may further comprise a suitable carrier. The *I. ricinus* salivary gland polypeptide vaccine composition is preferably administered orally or parenterally (including subcutaneous, intramuscular, intravenous, intradermal injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers,

bacteriostats and solutes which render the formulation iotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example; sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity to the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

Yet another aspect relates to an immunological/vaccine formulation which comprises the polynucleotide of the invention. Such techniques are known in the art, see for example Wolff *et al* , *Sciences*, (1990) *247* : 1465-8.

Another aspect of the invention related to the use of these *I. ricinus* salivary gland polypeptides as therapeutic agents. In considering the particular potential therapeutic areas for such products, the fields covered by these products are : haematology (particularly coagulation clinics), transplantation (for immunosuppression control), rheumatology (for anti-inflammatories), and general treatment (for specific or improved anaesthetics).

Table 1: Sequences identified in the subtractive and the cDNA full-length libraries

	Motifs	Similar sequences in databases	Score	Class
Seq.1		No significative identity		III
Seq.2		No significative identity		III
Seq.3		No significative identity		III
Seq.4		No significative identity		III
Seq.5	Prokaryotic mbre lipoprotein lipid attachment site	No significative identity		III
Seq.6		R. melioti Nitrogen fixation (fixF)	0.00089	III
		Human Apolipoprotein B-100	0.0045	III
		Hu.mRNA for cAMP response element (CRE-BP1) binding prot	0.057	III
Seq.7	Kunitz family of serine protease inhibitor	Human BAC clone GS345D13	$4,7^{13}$	I
		H. sap Tissue factor Pathway Inhibitor	4^{-12}	I
		PRESENT INVENTION-2		
Seq.9	Prokaryotic mbrne lipoprotein lipid attachment site	No significative identity		III
Seq.10		Pea mRNA for GTP binding protection.	0.48	III
Seq.11		No significative identity		III
Seq.12		IL-11 R-Béta gene	0.18	II
Seq.13		No significative identity		III
Seq.14		C. gloeosporioides cutinase gene	0.082	III
Seq.15		No significative identity		III
Seq.16		Mouse Mrna for secretory protection cont. thranspondine motifs	0.014	III
Seq.17	Zinc dependent metallopeptidase family	B. jararaca mRNA for jararhagin	$1,1^{-5}$	I
		Agkistrodon contortrix metalloproteinase precursor	$3,9^{-5}$	I
Seq.19		O. aries gene for ovine INFRINGEMENT-alpha	0.7	II
		Interferon-omega 45	0.88	II
		Interferon-omega 20	0.89	II
		RCPT PGE2	0.85	III
		PGE Rcpt EP2	0.85	III
Seq.20		No significative identity		III
Seq.21		IgG1L chain directed against human IL2	0.19	II
		rcpt Tac protection	0.2	II
		Var region of light chain of MAK447/179		
Seq.22		No significative identity		III
Seq.23		No significative identity		III
Seq.24		Mus Musculus neuroactin	0.42	III

Seq.25		No significative identity		III
Seq.26		H. sapiens thrombin inhibitor	$2,1^{-12}$	I
		Cycloplasmic antiproteinase 38kDa intracellular serine protection.	$2,3^{-12}$	I
Seq.28		No significative identity		III
Seq.29		No significative identity		III
Seq.30		Mus musculus transcription factor ELF3 (fasta)	0.053	III
Seq.31 28		Homo sapiens putative interferon-related protein (SM15) mRNA	1.70E-22	II
Seq.33		R. norvegicus Mrna for leucocyle common antigen-related protein	4.80E-09	II

(SEQ.ID.NO. 26 (Iris) : homology with H. sapiens thrombin inhibitor 2.1-12, class I

Class I : putative anticoagulant homologs ; Class II : putative immunomodulatory homologs ; Class III : low or no homologies found in the databases).

Table 2. Biological characteristics of the selected clones

Clone	Full-length sequences similarly to databases	Fasta/Blastp Scores[a]	ORF (aa)	Motifs	Signal peptide scores[b]	Sp length / Prob.	Nucleotide in position -3[c]
Seq31	Homo sapiens putative interferon-related gene (SKMc15) [U09585]	$1,8.10^{-36}/1.10^{-71}$	426		D 5,4/F [e]	48aa/8, 4.10^{-1}	G
Seq33	R.norvegicus leukocyte common antigen (LAR) mRNA [X83546]	$7,8.10^{-11}$/N	274		10,2/S	18aa/7, 4.10^{-3}	A
Seq17	Mouse mRNA for secretory protein containing thrombospondin motives [D67076]	$0,002/6.10^{-7}$	489	Metallo pep-tidase	7,9/S	19aa/7, 4.10^{-4}	G
Seq26	Pig leukocyte elastase inhibitor mRNA [P80229]	$0/7.10^{-67}$	378	Serpin	8,5/S	51aa/3, 28.10^{-3}	A
Seq7	Human Tissue Factor Pathway Inhibitor [P48307]	$4,8.10^{-12}/2.10^{-5}$	87	Kunitz	6,5/S	19aa:1, 8.10^{-4}	G

[a] No score (N)

[b] Succeeded (S) and Failed (F)

[c] Guanine (G) and Adenine (A)

[d] von Heijne analysis

[e] McGeoch analysis

Example 2: Construction of a Representational Difference Analysis (RDA) subtractive library.

The salivary glands of 5 day engorged or unfed free of pathogen *I. ricinus* female adult ticks were used in this work.

When removed, these glands were immediately frozen in liquid nitrogen and stored at -80°C. To extract RNA messengers (mRNA), the salivary glands were crushed in liquid nitrogen using a mortar and a pestle. The mRNAs were purified by using an oligo-dT cellulose (Fast Track 2.0 kit, Invitrogen, Groningen, The Netherlands). Two micrograms of mRNAs were extracted from 200 salivary glands of fed ticks, and 1.5 µg of mRNAs were also extracted from 1,000 salivary glands of unfed ticks.

All procedures were performed as described by Hubank and Schatz (1994). Double-stranded cDNAs were synthesised using the Superscript Choice System (Life Technologies, Rockville, Maryland USA). The cDNAs were digested with *Dpn*II restriction enzyme, ligated to R-linkers, amplified with R-24 primers (Hubank and Schatz, 1994), and finally digested again with the same enzyme to generate a "tester" pool consisting of cDNAs from salivary glands of fed ticks and a "driver" pool consisting of cDNAs from salivary glands of unfed ticks. The first round of the subtractive hybridisation process used a tester/driver ratio of 1:100. The second and third rounds utilised a ratio of 1:400 and 1:200,000, respectively. After three cycles of subtraction and amplification, the *Dpn*II-digested differential products were subdivided according to size into 4 different fractions on a 1.7% electrophoresis agarose gel, and subcloned the

*Bam*HI site of the pTZ19r cloning vector. The ligated product was used to transform TOP-10 *E. coli* competent cells (Invitrogen, Groningen, The Nederlands). Nine thousand six hundred clones of this subtractive library were randomly selected, and individually put in 96-well microplates and stored at -80°C. This subtractive library was analysed by sequencing 89 randomly chosen clones, using M13 forward and reverse primers specific to a region located in the pT19r cloning vector. The DNA sequences of these 89 clones were compared, and 27 distinct family sequences were identified. Homology of these sequences to sequences existing in databases is presented in Table 1.

The subtractive sequences 1 to 27 are presented in the sequence-listing file (except for sequences 17 and 26 whose complete mRNA sequences are presented ; see also Example 2). Three sequences (SEQ.ID.NO.7, 17 and 26) were selected for further characterization of their corresponding full-length mRNA sequence. These 3 sequences matched the sequence of i) the human tissue factor pathway inhibitor (TFPI), ii) a snake venom zinc dependent metallopeptidase protein, and iii) the human thrombin inhibitor protein, corresponding to SEQ.ID.NO.7, 17 and 26, respectively. These genes encode proteins which could be involved in the inhibition of the blood coagulation or in the modulation of the host immune response.

Example 3: Construction of the full length cDNA library and recovery of full length cDNAs sequences by screening of this full length cDNA library.

This library was set up using mRNAs extracted from salivary glands of engorged ticks. The mRNAs (80 ng) were subjected to reverse transcription using a degenerated oligo-dT primer (5'A(T)30VN-3'), the Smart™ oligonucleotide (Clontech, Palo Alto, USA), and the Superscript II reverse transcriptase (Life Technologies, Rockville, Maryland, USA). The single strand cDNA mixture was used as template in a hot start PCR assay including the LA Taq polymerase (Takara, Shiga, Japan), the modified oligo-dT primer and a 3'-Smart primer specific to a region located at the 5' end of the Smart™ oligonucleotide. The PCR protocol applied was: 1 min at 95°C, followed by 25 sec at 95°C/5 min at 68°C, 25 times; and 10 min at 72°C. The amplified double stranded cDNA mixture was purified with a Centricon 30 concentrator (Millipore, Bedford, USA). The cDNAs were divided into 4 fractions ranging from 0.3 to 0.6 kb, 0.6 to 1 kb, 1 kb to 2 kb, and 2 kb to 4 kb on a 0,8% high grade agarose electrophoresis gel. Each fraction was recovered separately by using the Qiaex II extraction kit (Qiagen, Hilden, Germany). The 4 fractions were ligated individually into the pCRII cloning vector included in the TOPO cloning kit (Invitrogen, Groningen, The Netherlands). The ligated fractions were then used to transform XL2-Blue ultracompetent *E. coli* cells (Stratagene, Heidelburg, Germany). The resulted recombinant clones were stored individually in microplates at -80°C. Ten clones were randomly chosen for partial or complete sequencing. As a result of this procedure, 2 cDNA sequences (SEQ.ID.NO.31 and SEQ.ID.NO.33, see Table 1) were selected for their homology to sequence databases. One is closely homologous to an interferon-like protein

(SEQ.ID.NO.31), whereas the other shows homologies to the *Rattus norvegicus* leukocyte common antigen-related protein (SEQ.ID.NO.33).

The 4 different fractions of the full-length cDNA library were screened with radio-labelled oligonucleotide probes specific to selected clones identified in the subtractive cDNA library. The labelling of these oligo probes was performed as described in "Current Protocols in Molecular Biology" (Ausubel et al, 1995, J. Wiley and sons, Eds). These 4 different fractions were then plated on nitrocellulose membranes and grown overnight at 37°C. These membranes were denatured in NaOH 0.2M/NaCl 1.5M, neutralised in Tris 0.5M pH 7.5-NaCl 1.5M and fixed in 2X SSC (NaCl 0.3 M/Citric Acid Trisodium di-hydrated 0.03 M). The membranes were heated for 90 min. at 80°C, incubated in a pre-hybridisation solution (SSC 6X, Denhardt's 10X, SDS 0,1%) at 55°C for 90 min., and finally put overnight in a preheated hybridisation solution containing a specific radio-labelled oligonucleotide probe at 55°C. The hybridised membranes were washed 3 times in a SSC 6X solution at 55°C for 10 min, dried and exposed on Kodak X-OMAT film overnight at -80°C.

The full length cDNA library was also analysed by sequencing a set of clones. The resulted DNA sequences were compared to EMBL/GenBank databases and were used to set up oligonucleotide probes to recover other corresponding clones. In this way, the complete consensus mRNA sequence of the SEQ.ID.NO.28 and 29 was confirmed by the recovery of two other clones corresponding to these sequences. Only one full-length cDNA clone corresponding to the subtractive clone 17 was isolated. Therefore, to identify the complete sequence of

the SEQ.ID.NO.17 and SEQ.ID.NO.26, the Rapid Amplification of cDNA Ends (RACE) method was applied.

The RACE methodology was performed as described by Frohman et al. (1995). The reverse transcription step was carried out using 10 ng of mRNAs extracted from salivary glands of engorged ticks and the Thermoscript Reverse transcriptase (Life technologies, Rockville, Maryland, USA). All gene specific primers (GSP) had an 18 base length with a 61% G/C ratio. The amplified products were subjected to an agarose gel electrophoresis and recovered by using an isotachophorese procedure. The cDNAs were cloned into the pCRII-TOPO cloning vector (Invitrogen, Groningen, The Netherlands). To identify the consensus cDNA sequence, different clones were sequenced., and their sequence was compared to their known corresponding sequence. Therefore, the complete cDNA sequences of the clones 17 and 26 isolated in the subtractive library were obtained by this RACE procedure (figure 1).

Example 4: Analysis of the full sequences of 5 selected clones.

The sequences of selected clones (SEQ.ID.NO.7, 17, 26, 31 and 33) allowed identification of the open reading frames, from which the amino sequence were deduced. These potential translation products have a size between 87 and 489 amino acids (see table 2). In order to evaluate, *in silico*, their respective properties, the amino acid sequences and the nucleotide sequences of said 5 open frames were compared with the databases using the tFasta and Blastp algorithms.

These comparisons show that SEQ.ID.NO.7 is highly homologous to the human Tissue Factor Pathway Inhibitor (TFPI). TFPI is an inhibitor of serine proteases having 3 tandemly arranged Kunitz-type-protease-inhibitor (KPI) domains. Each of these units or motifs has a particular affinity for different types of proteases. The first and second KPI domains are responsible for the respective inhibition of VIIa and Xa coagulation factors. The third KPI domain apparently has no inhibitory activity. It should be noted that the corresponding polypeptide sequence of SEQ.ID.NO.7 cDNA clone is homologous to the region of the first KPI domain of TFPI and that the KPI is perfectly kept therein. This similarity suggests that the SEQ.ID.NO.7 protein is a potential factor VIIa inhibitor.

The amino sequence deduced from the SEQ.ID.NO.28 clone has a great homology with 3 database sequences, namely : mouse TIS7 protein, rat PC4 protein and human SKMc15 protein. These 3 proteins are described as putative interferon type factors. They possess very well conserved regions of the B2 interferon protein. Therefore, it is proposed that the SEQ.ID.NO.31 protein has advantageous immunomodulatory properties.

Sequences SEQ.ID.NO.17 and SEQ.ID.NO.26 were compared with databases showing homology with the *Gloydius halys* (sub-order of ophidians) M12b metallopeptidase and the porcine elastase inhibitor belonging to the super-family of the serine protease inhibitors (Serpin), respectively. The amino sequences of these 2 clones also have specific motifs of said families. It is proposed that said proteins have advantageous anticoagulant and immuno-modulatory properties.

Finally, the SEQ.ID.NO.33 clone has a weak homology with the *R. norvegicus* leukocyte common antigen (LAR) that is an adhesion molecule. It is thus possible that the SEQ.ID.NO.33 protein has immunomodulatory properties related to those expressed by the LAR protein.

Due to their potential properties, most of the proteins examined are expected to be secreted in the tick saliva during the blood meal. Accordingly, tests were made for finding the presence of a signal peptide at the beginning of the deduced amino sequences. All of the results obtained with the Von Heijne analysis method were positive. By the McGeoch method, signal peptide sequences were detected for the SEQ.ID.NO.7, SEQ.ID.NO.17, SEQ.ID.NO.26 and SEQ.ID.NO.33 deduced amino sequences. It seems that said proteins are secreted in the tick salivary gland. Furthermore, the presence of a Kozak consensus sequence was observed upstream of the coding sequences of all examined clones. This indicates that their mRNAs potentially could be translated to proteins.

Example 5: Evaluation of the differential expression of the cDNA clones isolated in the subtractive and full-length cDNA libraries.

The differential expression of the mRNAs corresponding to the 5 full-length selected clones (SEQ.ID.NO.7,SEQ.ID.NO.17, SEQ.ID.NO.26, SEQ.ID.NO.31 and SEQ.ID.NO.33) and of 9 subtractive clones was assessed using a PCR and a RT-PCR assays (figure 2).

The PCR assays were carried out using as DNA template cDNAs obtained from a reverse transcription

procedure on mRNAs extracted from salivary glands either of engorged or of unfed ticks.

Each PCR assay included pair of primers specific to each target subtractive or cDNAs full-length sequence. PCR assays were performed in a final volume of 50 µl containing 20 pM primers, 0.2 mM deoxynucleotide (dATP, dCTP, dGTP and dTTP ; Boehringer Mannheim GmbH, Mannheim, Germany), PCR buffer (10 mM TrisHC1,50 mM KCI, 2.5 mM. MgC12, pH 8.3) and 2.5 U of Taq DNA polymerase (Boehringer mannheim GmbH, Mannheim, Germany).

DNA samples were amplified for 35 cycles under the following conditions: 94 C for 1 min., 72 C for 1 min. and 64 C for 1 min, followed by a final elongation step of 72 C for 7 min.

The RT-PCR assay was carried out on the 5 selected full-length cDNA clones and on 5 cDNA subtractive clones. The mRNAs used as template in the reverse transcription assay was extracted from salivary glands of engorged and unfed *I. ricinus* ticks. The reverse transcription assays were performed using a specific primer (that target one the selected sequences) and the "Thermoscript Reverse transcriptase" (Life technologies, Rockville, Maryland, USA) at 60°C for 50 min. Each PCR assay utilised the reverse transcription specific primer and an another specific primer. The PCR assays were performed in a final volume of 50 µl containing 1 µM primers, 0.2 mM deoxynucleotide (dATP, dCTP, dGTP and dTTP; Boehringer Mannheim GmbH, Mannheim, Germany), PCR buffer (10 mM Tris HCI, 50 mM KCl, 2.5 mM $MgCl_2$, pH 8.3) and 2.5 U of Expand High Fidelity polymerase (Roche, Bruxelles, Belgium). Single

stranded DNA samples were amplified for 30 cycles under the following conditions: 95°C for 1 min., 72°C for 30 sec. and 60°C for 1 min, followed by a final elongation step of 72°C for 7 min.

The figure 2 shows that the expression of the selected sequences is induced in salivary glands of 5 day engorged ticks, except for the sequence 31 that is expressed at a similar level in salivary glands of engorged and unfed ticks. The expression of the other mRNAs could be either induced specifically or increased during the blood meal.

Example 6: Expression of recombinant proteins in mammal cells.

The study of the properties of isolated sequences involves the expression thereof in expression systems allowing large amounts of proteins encoded by these sequences to be produced and purified.

The DNA sequences of the 5 selected clones (SEQ.ID.NO.7, SEQ.ID.NO.17, SEQ.ID.NO.26, SEQ.ID.NO.31 and SEQ.ID.NO.33) were transferred into the pCDNA3.1 His/V5 expression vector. Said vector allows the expression of heterologous proteins fused to a tail of 6 histidines as well as to the V5 epitope in eukaryotic cells. The different DNAs were produced by RT-PCR by using primers specific to the corresponding clones. These primers were constructed so as to remove the stop codon of each open reading frame or phase in order to allow the protein to be fused to the 6xHIS/Epitope V5 tail. In addition, the primers contained restriction sites adapted to the cloning in the expression vector. Care was

taken to use, when amplifying, a high fidelity DNA polymerase(Pfu polymerase, Promega).

The transient expression of the Seq16 and 24 recombinant proteins was measured after transfection of the Seq16 and Seq24-pCDNA3.1-His/V5 constructions in COS1 cells, using Fugen 6 (Boehringer). The protein extracts of the culture media corresponding to times 24, 48 and 72 hours after transfection were analysed on acrylamide gel by staining with Coomassie blue or by Western blot using on the one hand an anti-6x histidine antibody or on the other hand Nickel chelate beads coupled to alcaline phosphatase.

These analyses showed the expression of said proteins in the cell culture media.

Example 7: Expression of proteins in *E. coli*

7.1. Insertion of coding sequences into the pMAL-C2E expression vector.

Proteins fused with the Maltose-Binding-Protein (MBP) were expressed in bacteria by using the pMAL-C2E (NEB) vector. The protein of interest then could be separated from the MBP thanks to a site separating the MBP from the protein, said site being specific to protease enterokinase.

In order to express optimally the 5 sequences examined, using the pMAL-C2E vector, PCR primer pairs complementary to 20 bases located upstream of the STOP codon and to 20 bases located downstream of the ATG of the open reading frame or phase were constructed. The amplified cDNA fragments only comprise the coding sequence of the target mRNA provided with its stop codon. The protein of interest

was fused to MBP by its N-terminal end. On the other hand, since these primers contained specific restriction sites specific to the expression vector, it was possible to effect direct cloning of the cDNAs. The use of Pfu DNA polymerase (Promega) made it possible to amplify the cDNAs without having to fear for errors introduced into the amplified sequences.

The coding sequences of clones SEQ.ID.NO.7, SEQ.ID.NO.17, SEQ.ID.NO.26 and SEQ.ID.NO.31 were reconstructed in that way. Competent TG1 cells of *E. coli* were transformed using these constructions. Enzymatic digestions of these mini-preparations of plasmidic DNA made it possible to check that the majority of clones SEQ.ID.NO.7, SEQ.ID.NO.17, SEQ.ID.NO.26 and 31-p-MALC2-E effectively were recombinant.

7.2. Expression of recombinant proteins.

Starting from various constructions cloned in TG1 *E. coli* cells, the study of the expression of recombinant proteins fused with MBP was initiated for all sequences of interest (*i.e.* SEQ.ID.NO.7, SEQ.ID.NO.17, SEQ.ID.NO.26 and SEQ.ID.NO.33) except for SEQ.ID.NO.31. The culture of representative clones of SEQ.ID.NO.7, SEQ.ID.NO.17, SEQ.ID.NO.26 and SEQ.ID.NO.33 as well as negative controls (non recombinant plasmids) were started to induce the expression of recombinant proteins therein. These cultures were centrifuged and the pellets were separated from the media for being suspended in 15 mM pH7.5 Tris and passed through the French press. The analysis of these samples on 10% acrylamide gel coloured with Coomassie blue or by Western

Blot using rabbit anti-MBP antibodies, showed the expression of recombinant proteins SEQ.ID.NO.7 (~50kDa), SEQ.ID.NO.17 (~92kDA), SEQ.ID.NO.26 (~80 kDA) and SEQ.ID.NO.31 (-67kDa).

Example 8: Production of antibodies.

The SEQ.ID.NO.7, SEQ.ID.NO.17 and SEQ.ID.NO.26 protein were injected into groups of 4 mice with the purpose of producing antibodies directed against said proteins. The antigens were firstly injected with the complete Freund adjuvant. Two weeks later, a recall injection was made with incomplete Freund adjuvant. The sera of mice injected with SEQ.ID.NO.17 provided positive tests for anti-MBP antibodies.

Example 9: Iris protein characterization

One clone, formerly named SEQ.ID.NO.26, was selected for further characterization of its recombinant protein, due to its similarity to the human thrombin inhibitor gene.

Using the RACE method, its complete cDNA sequence [Accession no: AJ269658] was recovered, and the complete ORF encodes a protein of 378 amino acids in length. Its comparison to EMBL/GenBank databases showed a high homology to the pig leukocyte elastase inhibitor, which shares a specific serine protease inhibitor motive. Based on the results, the Inventors have decided to call the tick protein SEQ.ID.NO.26 "Iris", for "*Ixodes Ricinus* Immunosuppressor".

Biological materials.

Salivary glands of unfed (n = 300) or 5 day engorged (n = 70) pathogen free *I. ricinus* female adult ticks were collected by teasing them away from other internal organs. The salivary glands were crushed in an extraction buffer (PBS 1X, pH 7,4; EDTA 10 mM, AEBSF 1mM - Sigma-Aldrich, Bornem, Belgium) for 10 min by using a potter and a pestle. The samples were centrifuged at 10,000g for 8 min, and the supernatants were recovered and stored at -20°C.

Saliva was collected from 5 day engorged adult female ticks by using a finely drawn capillary tube fitted over the mouthparts of each tick. Before collecting saliva, each tick was washed with PBS 1X pH 7.2, and was injected with PBS 1X pH 7.2 containing a 0.2% dopamine (Sigma-Aldrich, Bornem, Belgium).

Production of recombinant Iris proteins (rIris) in bacterial and mammalian expression systems.

The screening of a RDA subtractive library identified *iris* cDNA. This library was constructed as described by Hubank and Schatz (Hubank *et al*, 1994) by using RNA messengers extracted from unfed and 5 day engorged tick salivary glands. The complete *iris* cDNA sequence was recovered by performing the RACE methodology as described by Frohman (Frohman, 1995). Two recombinant Iris proteins were synthesised: one in fusion with the maltose binding protein (rIris/MBP), and one in fusion with V5/His EpiTag (rIris/His). The recombinant rIris/MBP protein was expressed in *E. coli* by using the pMALC2-E vector (NEB, Hitchin, UK). The rIris/His protein was expressed in CHO-KI cells by using

the pCDNA3.1/V5-His A vector in frame with the V5/His 6X EpiTag.

The rIris/His protein was also purified in batch by Ni-chelate chromatography (Ni-NTA superflow resin - Qiagen, Hilden, Germany) following the manufacturer's guidelines. Different buffers were used to purify the rIris/His protein: the lysis buffer (PBS 1X, NaCl 500 mM, Zwittergent 3.12 0.1%, pH7.5); the washing buffer (PBS 1X, NaCl 500 mM, Zwittergent 3.12 0.1%, imidazole 17.25 mM, pH 7.5), and the elution buffer (PBS 1X, NaCl 500 mM, Zwittergent 3.12 0.1%, imidazole 103 mM, pH 7.5). The eluate was dialysed (in a 7,000 Da cut-off membrane) in PBS 1X, NaCl 500 mM, pH 7.5. The concentration of rIris/His was evaluated on a Commassie blue stained acrylamide gel at ~10 ng/µl (250 nM), by comparison with known quantities of BSA.

Ten week-old female Balb/c mice were immunized with 5 µg of Seq.24/MBP in Freund's complete adjuvant. Three booster immunisations were carried out with the same amount of antigen in Freund's incomplete adjuvant, at 15-day intervals.

To examine the expression of native proteins in salivary glands and to detect rIris/His, the same quantities of fed and unfed tick salivary glands, and a rIris/His sample were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with diluted sera directed against rIris/MBP (1:1,000) and revealed with NBT-BCIP.

I. ricinus salivary glands were isolated from unfed, 3 day and 5 day fed ticks, and were immobilised on silanated slides (Biorad, Nazareth EKE, Belgium). Salivary

glands were fixed in a 4% paraformaldehyde solution for 30 min at room temperature. After a treatment with 0.5% Triton X-100, the samples were incubated in PBS 1X containing 5% FCS. The anti-rIris/MBP serum was used at a 1:10 dilution, and the secondary antibody, a FITC Anti-Mouse IgG (H+L) (ICN, Asse-Relegem, Belgium) at a 1:32 dilution. The slides were mounted in Vectashield mounting medium (Vector Lab, Peterborough, UK) and observed with a Leica confocal laser microscope by using a Leica TCS 4D operating system (Leica, Wetzlar, Germany).

Preparation of rIris/His cellular and rIris negative control (NEG) extracts for immune tests.

CHO-KI cells expressing rIris/His protein, obtained from a confluent culture in five 150 cm^2 flasks, were suspended in 1 ml of RPMI-1640 complete medium. The sample was frozen and thawed 3 times before being centrifuged at 50,000 g for 1 hour at 4°C. The supernatant was recovered and used in the different activity tests. The negative control (NEG) was a proteinic extract of CHO-KI cells resistant to G418 that do not express the recombinant protein and prepared as the rIris/His extract. The concentration of rIris/His in the cellular extract was evaluated at ~4 ng/µl (~100 nM), by comparing rIris/His contained in cellular extracts and purified rIris/His, on Western blot revealed with anti-V5 antibody (Invitrogen, Groningen, The Netherlands). The toxicity of the different samples for human PBMCs was evaluated by using the 7-AAD viability dye (Immunotech, Marseille, France), according to manufacturer's instructions.

Normal Balb/c spleen cells (SC):

A suspension of SC was obtained from normal Balb/c mice. 10^6 lymph node cells per well were cultivated for 2 hours in 100 µl of culture medium (RPMI-1640 (Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum (v/v), 2mM L-glutamin, 1mM sodium pyruvate, 1mM non-essential amino acids (Sigma, St Louis, MO), 0,05 mM mercaptoethanol, 100 U/ml penicillin/streptomycin (Gibco, Basel, Switzerland) and 25 µg/ml Funigizone - Gibco, Basel, Switzerland), with various dilutions of either rIris/His or NEG cellular extracts. Cells were stimulated with 10 µl of ConA (20 µg/ml) in a final volume of 200 µl for 15 hours. One µCi/well of [^{3}H]thymidine (Amersham Int., Amersham, UK) was added 24 hours before harvesting the cells. Tritiated thymidine incorporation was determined by liquid scintillation counting. Results showed the means of duplicate rIris/His or NEG stimulated wells realised in 2 independent experiments (+/- S.D.). Means of ConA-unstimulated wells were previously subtracted (net 10^3 c.p.m.).

Preinfested Balb/c axiliary and brachial lymph nodes cells:

Axilary and brachial lymph nodes were removed from a mouse killed 9 days after infestation with 15 pathogen-free *I. ricinus* nymphs. 10^6 lymph nodes cells were cultured for 2 hours in 100 µl of complete RPMI-1640 medium. After 96 hours of incubation with various dilutions of rIris/His or NEG samples, 1 µCi/well of [^{3}H]thymidine (Amersham Int., Amersham, UK) was added 18-24 hours before

harvesting the cells. Tritiated thymidine incorporation was determined by liquid scintillation counting.

Normal human PBMCs :

Experiments were done with PBMCs obtained from 8 different donors. Cells were resuspended in RPMI-1640 medium supplemented with FCS 10% (v/v), L-glutamine 2 mM, penicilline-streptomycine (100U/ml) and IL-2 (20 U/ml). 2.0 10^6 cells were pre-cultivated in 1 ml of culture medium. The cells were diluted at different concentrations in 96 wells plates : 2.0 10^5 cells/100 µl for Protein Purified Derivative (PPD) stimulation, 5.0 10^4 cells/100 µl for Lipopolysaccharides (LPS) stimulation for the ELIspot technique, and 2.0 10^5 cells/100 µl for the ELISA technique. Finally, PBMCs were incubated during 72 hours at 37°C with various dilutions of rIris/His or NEG, in the presence or not of anti-rIris/MBP serum and different activators : Phytohaemmaglutinnin (PHA - at a final concentration of 1 µg/ml), LPS (1 µg/ml), CD3/CD28, (500 ng/ml), Phorbol Myristate Acetate (PMA)/CD28 (PMA 25 ng/ml - CD28 500 ng/ml) PPD (5 µg/ml).

ELIspot assay :

96 wells nitrocellulose bottom coated plates (Multiscreen-HA Mahan, Millipore, Brussels, Belgium) were coated with coating antibodies directed against IFN-γ (clone C1-D16 MAB 1-D1K, Nodia, Antwerp, Belgium) and IL-10 (Clone JES3-9D7, BD Pharmingen, San Diego, CA). Cells were stimulated with PHA or LPS and S24p or S24n for 72 hours at 37°C. Supernatants were recovered and conserved at -20°C

before being analysed. The cytokines were detected with biotinylated anti-IFN-γ antibody (clone JES3-5A10 MAB 7-B6-1, Nodia, Antwerp, Belgium) and anti-IL10 antibody (clone JES3-12G8, BD Pharmingen, San Diego, CA) diluted in PBS Tween 0,25% (1µg/ml). Finally, the plates were incubated with extravidine peroxydase and AEC substrate (Sigma-Aldrich, Bornem, Belgium). Results show the means of triplicate rIris/His or NEG cellular extracts stimulated wells (+/- S.D.). Means of unstimulated wells were previously subtracted.

ELISA technique :

The different cytokines-specific ELISA were performed to detect of IFN-γ, IL-10, TNF-α, IL-6, IL-1β and IL-8. This was done by using the Flexia-human kit (Biosource, Nivelles, Belgium). In the case of the detection of IL-5, the IL-5 kit (Endogene, Woburn, Massachusetts) was used. The assays were carried out using manufacturer's instructions and were revealed using TMB substrate. The concentration of the different cytokines (pg/ml) was calculated by comparison to a standard curve generated with the different cytokines. Results show the means of rIris/His or NEG stimulated wells realised in 5 independent experiments (+/- S.D.). Means of unstimulated wells were previously subtracted.

Example 10: Detection of Iris in *I. ricinus* salivary glands and saliva.

Two recombinant Iris proteins were expressed either in *E. coli* cells using the pMALC2-E vector (NEB, Hitchin, UK) in fusion with the maltose binding protein (MBP)

leading to the expression of a rIris/MBP 82 kDa fusion protein (Figure 3) or in CHO-K1 cells by using the pCDNA3.1/V5-His A vector resulting in the expression of a rIris/His Tag fusion protein with a Mr of 43 kDa (Figure 3). Immune sera recovered from mice injected with rIris/MBP were used to detect both rIris/His in CHO-K1 cells and the corresponding native Iris protein in unfed, in 3 day and 5 day fed female *I. ricinus* salivary glands. Recombinant and native proteins were detected on Western blots (Figure 3). A similar double band pattern was revealed at ~46 kDa and 40 kDa (rIris/His) and at ~43 kDa and 40 kDa (native Iris) in CHO-KI extracts and in 5 day fed tick salivary glands, respectively. Interestingly, the protein was not detected in unfed tick salivary glands. Moreover, Iris was revealed in tick saliva at a molecular weight of 43 kDa.

By using confocal microscopy, Iris was found in 3 day and, more abundantly, in 5 day fed tick salivary glands on the external surface of salivary acini, within the cells and also in the acini's light; but was not detected in unfed tick salivary glands (Figure 4). All of these results infer that the expression of Iris is induced in the salivary glands during the tick feeding process and that Iris is secreted in tick saliva.

Example 11: Characterization of the immunomodulatory properties of Iris.

Based on its homology to a neutrophil elastase inhibitor, the immunomodulatory properties of Iris were examined by using different activity tests that were mainly performed with soluble proteinic extracts of CHO-KI cells

expressing rIris/His at a concentration of ~4 ng/µl (100 nM). Proteinic extracts of CHO-KI cells, which do not express rIris/His, were used as a negative control (NEG).

In vitro proliferation of Balb/c normal spleen cells and tick-specific lymph nodes cells.

The proliferation of normal Balb/c spleen cells (SC) was analysed in vitro by pre-incubating them with various dilution (1:6.25 to 1:50) of rIris/His cellular extracts, followed by stimulation with concanavaline-A (ConA). As shown on figure 5, the proliferative response of SC was strongly diminished (81% at dilution 1:6.25) in dose dependent concentration. The negative control had no significant effect on ConA-stimulated SC (average inhibition of 15%); even if this inhibited by 25% the SC proliferation at a 1:6.25 dilution.

The immunogenicity of Iris was also studied by evaluating the proliferative responses of draining lymph node cells (LC) from one Balb/c mouse that was infected with 15 pathogen-free nymph *I. ricinus*. The isolated LC were incubated with increasing amount of both rIris/His and NEG protein extracts. The results indicated that in the presence of rIris/His, the proliferation of these LC was strongly inhibited in a dose dependent concentration (inhibition by 98.5 % at dilution 1:6.25 in comparison to dilution 1:25, see figure 6). In contrast, the NEG protein extract had no significant effect on LC proliferation.

Example 12: In vitro cytokine production by human PBMCs.

The effect of rIris/His on cytokine production was studied on human peripheral bone marrow cells (PBMCs) stimulated with different activators. The number of PBMCs secreting IFN-γ and IL-10, after stimulation either with lipopolysaccharides (LPS) or phytohaemagglutinin-A (PHA), was assayed by the ELISPOT technique (Figure 7). Under PHA stimulation, in presence of rIris/His cellular extract, a reduced number of PBMCs (more than 80%) expressed IFN-γ while the number of cells producing IL-10 remained unchanged. The NEG protein extract had not effect on the production of both cytokines by PHA-stimulated PBMCs. In contrast, after LPS stimulation, no difference in the number of cells producing IFN-γ was observed between PBMCs incubated with rIris/His and the NEG cellular extract. On the other hand, rIris/His extract enhanced by 400% the number of PBMCs expressing IL-10, while stimulation with NEG cellular extract inhibited by 77% the number of cells producing IL-10.

The effect of the rIris/His cellular extract (used at a 1:5 dilution) on the production of cytokines (IFN-γ, IL-6, TNF-α, IL-10, IL-8 and IL-1β) by PBMCs stimulated with a set of activators (PHA, CD3/CD28, PMA/CD28, LPS and PPD) was also evaluated by ELISA (Table 3).

Table 3: Cytokine production by PBMC treated with rIris/his or NEG cellular extracts

	Cell Stimulation					
	PHA	CD3/CD28	PMA/CD28	LPS	PPD	
IFN-γ	36 92	46 145	8 157	43 64	6 101	rIris/His NEG
expression	-	-	-	/	-	
IL-6	14 88	8 95	75 84	10 195	7 61	rIris/His NEG
expression	-	-	/	-	-	
TNF-α	12 38	525 108	53 181	10 89	15 52	rIris/His NEG
	-	+	-	-	-	
IL-10	- -	88 101	- -	116 27	130 98	rIris/His NEG
expression	/	/	/	+	/	
IL-8	5 54	7 22	- -	0 60	0 66	rIris/His NEG
expression	-	/	/	-	-	

5 Values represent % of cytokine production calculated in comparison with cells stimulated only with the activator. Expression is: inhibited (-), enhanced (+), unchanged or undefined (/).

The results indicate that the production of almost all tested cytokines (IFN-γ, IL-6, TNF-α, IL-10, and IL-8) was inhibited by the rIris/His cellular extract, except for the IFN-γ production that was unaffected after LPS stimulation. Moreover, the production of IL-10 was not modulated after treatment with almost all activators, except under LPS stimulation, which slightly enhanced IL-10 production. In contrast, the NEG cellular extract had no significant effect on the cytokine production, except after LPS stimulation that inhibited the IL-10 production. Furthermore, it was shown that IL-1β production was unaffected by rIris/His cellular extract. The dose dependent effect of rIris/His was examined by analysing the IFN-γ, IL-5 and IL-10 production under CD3/CD28 and PPD stimulation (Figure 8). In these cases, the maximum inhibition of IFN-γ production by rIris/His was of ~65% (P<0.01) and ~75% (P<0.05) after CD3/CD28 and PPD stimulation, respectively. This inhibition was still effective at a 1:12.5 dilution. In contrast, no difference in the production of IL-5 and IL-10 was observed between PBMCs incubated with rIris/His and NEG cellular extracts.

To confirm the role of Iris in the modulation of the production of some cytokines, PBMCs stimulated by CD3/CD28 activator were incubated with various dilutions (from 1:250 to 1:4,000) of anti-rIris/MBP serum (Figure **9a**). It was observed that PBMCs treated with rIris/His cellular extract (at dilution 1:12.5) in the presence of anti-rIris/MBP serum restored the IFN-γ production in a dose dependent manner (Figure 9a); whilst this antiserum itself

had no immuno-stimulating effect on cytokine production by the PBMCs.

At dilution 1:250, the antiserum re-established the IFN-γ production to a level similar to that obtained by CD3/CD28-stimulated PBMCs without the presence of rIris/His.

To assert the specificity of the neutralising activity of the anti-rIris/MBP serum, its effect was measured on the activity of cyclosporine-A (CsA) (Figure 9b), an immunosuppressive drug. The effect of a serum specific to an unrelated MBP fusion protein was also measured on rIris/His cellular extracts activity (Figure 9a). The anti-rIris/MBP serum (at a 1:250 dilution) did not affect the activity of 400 nM CsA, and the unrelated antiserum had no effect on the rIris/His immunomodulatory activity.

Finally, a small amount of rIris/His was purified from the rIris/His CHO-K1 cellular extract (Figure **9c**). This purified rIris/His protein, at a 25 nM concentration, completely inhibited the IFN-γ production by CD3/CD28-stimulated PBMCs, which was partially restored (50 % of the IFN-γ production by CD3/CD28-stimulated PBMCs in absence of rIris/His) by using the anti-rIris/MBP serum at 1:250 dilution. Interestingly, it was found that the level of inhibition of ~25 nM of purified rIris/His was comparable to that of 400 nM CsA. The incubation of CD3/CD28-stimulated PBMCs with either a purified NEG or the anti-rIris/MBP (at 1:250 dilution) had no influence on the IFN-γ production.

It is now well established that the modulation of host immunity by tick saliva is of major importance in the successful accomplishment of the blood meal and in the transmission of tick-borne pathogens such as Borrelia

burgdorferi, the causal agent of Lyme disease (Zeidner et al, 1996).

Although extensive information is available on the effects of tick feeding on host immune defenses, little is known about the nature of the immunomodulatory molecules expressed by tick salivary glands.

Tick salivary gland extracts (SGE) modulates host immune response by modifying the activity of several immune cells (lymphocytes, monocytes, macrophages, ...). An example of this is the inhibition of T lymphocyte proliferation in response to mitogens (Wikel, 1982) and the production of Th1 cytokines as IFN-γ and IL-2 by SGE (Ramachandra et al, 1992).

Moreover, Th2 type cytokine production such as IL-10, IL-5 and IL-4 is enhanced or remains unchanged (Ganapamo et al, 1995) (Ganapamo et al, 1996).

Tick SGE also inhibits the production of several cytokines (IFN-γ, IL-8, IL-6, TNF-α,...) by human peripheral blood lymphocytes stimulated with LPS (Fuchsberger et al, 1995). Some studies indicated that these phenomena are induced by proteins (Urioste et al, 1994); (Schoeler et al, 2000); (Bergman et al, 2000).

The present invention has characterised the properties of a protein induced during the tick feeding process, which is called Iris for "Ixodes ricinus immunosuppressor", due to its exceptional properties. The corresponding mRNA sequence was first recovered by analysing a RDA subtractive library, and by using the RACE method.

In order to determine the immunomodulatory properties of Iris, the Inventors have studied the effect of

the corresponding recombinant protein (rIris/His) on normal Balb/c spleen and lymph node cell proliferation, and on human PBMCs cytokine production, using specific T-lymphocytes (PHA, ConA, CD3/CD28 and PMA/CD28), macrophages (LPS) and antigen presenting cell - APC (PPD) activators. The results indicated that rIris/His cellular extracts inhibited the proliferation of murine lymphocytes on a dose dependent manner.

ELISA and ELIspot assays showed that the rIris/His cellular extracts suppressed the production of IFN-γ by TL and APC, while IL-5 and IL-10 level remained stable. In contrast, rIris/His extract did not affect IFN-γ production and enhanced the expression of IL-10 by macrophages.

It was also shown that the expression of the pro-inflammatory cytokines IL-6 and TNF-α by macrophages, TL, and APC was inhibited, while IL-1β expression remained unaffected.

Furthermore, by neutralising completely rIris/His cellular extract activity with a specific anti-rIris serum, and by showing that purified rIris/His protein inhibited IFN-γ production by T cells, it has been clearly established that the recombinant protein was effectively responsible of the immunomodulation.

Importantly, the inhibitory effect of ~25 nM rIris/His on IFN-γ production (inhibition of 94%) is comparable to 400 nM CsA activity (inhibition of 99%).

These observations indicate that Iris is a novel immunosuppressor secreted by *I. ricinus* salivary glands into the saliva during the blood meal. It suppresses T lymphocyte proliferation and induces a Th2 type immune

response that is characterised by the inhibition of IFN-γ production and an unaffected expression of IL-5 and IL-10. In addition, Iris modulates the mechanisms of innate immunity by inhibiting the production of pro-inflammatory cytokines (IL-6 and TNF-α).

It is known that several immunomodulatory factors are secreted in saliva at various times of the feeding process. Indeed, it was shown that SGE prepared daily from engorging ticks suppressed IL-1 production from day 0 to day 5 of engorgement while TNF-α production was suppressed during the entire blood meal (Ramachandra et al, 1992).

For this reason, it is suggested that Iris and other factors modulate host immunity at day 3 of engorgement. In contrast, from day 5 of engorgement, Iris is the only or the most important immunomodulatory factor, contained in tick saliva.

Finally, tick induced inhibition of IL-2, TNF-α and IFN-γ appears to facilitate *B. burgdorferi* survival in the vertebrate host, during the early phase of infection (Zeidner et al, 1996).

For this reason, based on its immune properties, Iris could be considered a major salivary factor that facilitates *B. burgdorferi* transmission.

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